

Emerging Homogeneous DNA-based Technologies in the Clinical Laboratory

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Background: Advances in molecular diagnostic technologies have enabled genetic testing in single closed-tube reactions. The purpose of this review is to highlight some of the platforms and technologies currently available for the homogeneous detection of targets and the application of the technologies in the clinical setting. Validation issues surrounding the technologies, which may need to be addressed before they can become widely accepted, will also be discussed.

Approach: This review discusses the principles of several of the major technologies available for performing homogeneous genetic analyses. Publications arising from the application of the technologies in a wide range of clinical areas are used to highlight and compare the potential advantages and shortcomings of the various technologies.

Content: This review is descriptive and focuses on three areas: the technologies available for performing homogeneous analysis, the clinical applications where the technologies are being used, and validation issues surrounding the acceptance of the technologies in the general clinical setting.

Summary: This review intends to give the reader a greater understanding of the various technologies available for performing homogeneous genetic testing in the clinical laboratory. Through insight into the principles and performance characteristics underlying these technologies, the end user can evaluate their value and limitations in the clinical diagnostic setting.

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DNA-based analyses are currently used routinely in a wide range of clinical settings, including molecular genetics, oncology, microbiology, hematology, blood transfusion, and immunology. Many of the techniques currently in use rely on PCR amplification of a target molecule

followed by a variety of postamplification analyses, such as restriction enzyme digestion and agarose gel electrophoresis, to detect the specific analyte amplified.

Quality issues surrounding the applicability, reliability, and reproducibility of the more established DNA-based techniques in use in the clinical laboratory have been highlighted previously (1, 2). Key factors include sample quality and preparation, the sensitivity of *Taq* polymerase to inactivating contaminants, amplification bias and variability, the selection of appropriate positive and negative controls, restriction enzyme digestion efficiency and reproducibility, and the cross-contamination of PCR amplifications.

Advances in DNA analysis to develop methods, which are increasingly specific, sensitive, fast, simple, automatable, and cost-effective, are considered paramount. These demands are currently driving the rapid evolution of a diverse range of newer technologies. Clearly, some of these rapidly developing technologies will offer numerous advantages and benefits to the services offered by clinical laboratories.

One such recent advance is the development of technologies with the ability to amplify, detect, and quantify DNA targets in a single closed-tube reaction as PCR proceeds. These real-time homogeneous systems offer many advantages over traditional methods, including speed, reduced risk of contamination, and the ability to more accurately quantify the amount of starting material present. These technologies have been used for the on-line quantification of nucleic acid targets, mutation detection, and sequence confirmation. These are used primarily in basic and clinical research studies in oncology, genetic diseases, and infectious diseases; in gene expression studies; and in genetic diagnostics.

As rapid technological advances produce systems that are capable of increased sensitivity, throughput, and quantitative potential, new sets of validation issues need to be addressed. These include the reproducibility of the techniques, both intra- and interlaboratory, in terms of sensitivity, accuracy, precision, and the interpretation of data where arbitrary selection of cutoff points to separate positive and negative results are often used.

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There is a need for the parallel validation of these technologies as they make the transition from the research laboratory to the clinical laboratory and for the development of tools to enhance validity, such as suitable reference calibrators and internal/external quality evaluations.

In the United Kingdom and Europe, schemes such as the UK National External Quality Assessment Scheme (UKNEQAS) (3) and the European Molecular Genetics Quality Network (EMQN) (4) are going some way to addressing these issues.

This report will highlight some of the most popular homogeneous technologies that are currently making the transition from the research laboratory to the clinical laboratory and discuss key aspects in their validation.

Background

Homogeneous methods are essentially single-tube assays in which all of the processes required for analyte amplification and detection occur in a single "closed-tube" reaction. Combining the thermal cycling system with the signal detection system makes on-line monitoring of the PCR amplification process possible.

The advantages of homogeneous methods include the reduced risk of cross-contamination and the speed and simplicity of the methods, for which no post-PCR manipulations are required. The methods are generally amenable to automation and high-throughput processing with 96-well plates, the current industry standard, being rapidly replaced with 384-well plate capabilities. Maximum automation can be achieved using fully integrated systems with robotic processing of 96- or 384-well plates throughout the stages of DNA extraction, PCR set-up, amplification, detection, and analysis.

In addition, because the methods commonly allow for "real-time" monitoring of the entire PCR reaction, quantitative analyses are possible. The signal detected directly correlates with the amount of PCR product accumulating, which in the log-linear phase of the amplification process is dependent on initial target copy number. Performing measurements in the log-linear phase of amplification enables more accurate quantification than was previously possible with traditional gel-based techniques that examine end-point reactions.

Non-PCR-based technologies such as the ligase chain reaction and strand displacement amplification are also amenable to homogeneous detection methods. These newer technologies are currently less widely used in the general clinical setting than PCR, which will be the focus of this review.

Platforms

There are several platforms available for performing real-time PCR analyses. These range from ultrarapid, heated-air thermocyclers where PCR is performed in glass capillaries (Roche LightCycler) to tube and microtiter plate-based systems (ABI Prism 7700 Sequence Detection System).

The LightCycler utilizes a blue-light-emitting diode with fluorescence measured by three photodetection diodes with different wavelength filters. The capillaries are contained within a carousel, which is rotated past the diodes. The ultrarapid heating and cooling of the capillaries in the airstream allows amplifications to be completed in typically <20 min (5).

The ABI Prism 7700 Sequence Detection System utilizes an array of optical fibers to distribute the laser light across all 96 samples contained in either thin-walled tubes or microtiter plates. Emitted fluorescence returns via the fibers to a spectrograph with a charged coupled device camera. Fluorescence between 500 and 660 nm can be detected. The built-in thermal cycler typically takes ~2 h to complete amplification of the reactions.

As the use of real-time PCR assays gains popularity, a range of alternative real-time instruments are becoming available, including the iCycler (Bio-Rad), the Sentinel (Stratagene), and the SmartCycler (Cepheid).

Platforms available for non-PCR-based techniques include the BD ProbeTecET system (6), which is based on strand displacement amplification, and the Abbott LCx system, which is based on the ligase chain reaction (7).

Chemistries

There are a host of homogeneous technologies currently available for DNA analysis, all of which, in the broadest sense, rely on the same general principle that a change in the properties of a signal molecule occurs when the target analyte is present. The detection software charts signal accumulation once sufficient target has been produced to pass the detection threshold of the system. The point in the PCR at which the signal first begins to be detected above background is called the threshold cycle (C_t)¹ and is inversely proportional to the amount of starting material present in the reaction. The current chemistries used to monitor the amplification process include nonspecific double-stranded DNA (dsDNA)-binding dyes and specific probe hybridization-based systems.

NONSPECIFIC DETECTION METHODS

Dyes such as SYBR Green produce enhanced fluorescence signals upon binding to dsDNA duplexes. Increases in signal are monitored in real time as the dye binds to the newly synthesized DNA molecules as PCR proceeds. Signals are measured at the end of either the annealing or elongation step of each PCR cycle.

Although dsDNA-binding dyes are a simple, fast, and inexpensive way of monitoring amplicon production, the major disadvantage of the method is that the dye binds

¹ Nonstandard abbreviations: C_t , threshold cycle; dsDNA, double-stranded DNA; FRET, fluorescence resonance energy transfer; T_m , melting temperature; HBV, hepatitis B virus; CMV, cytomegalovirus; CMM, COBAS Amplicor CMV monitor; CML, chronic myelogenous leukemia; and SNP, single nucleotide polymorphism.

nonspecifically to all dsDNA such that primer-dimer species and nonspecific amplification products cannot be distinguished from the specific amplicon. Careful assay optimization to minimize the production of nonspecific species is critical.

Most platforms offer the opportunity to "melt" the amplicons after amplification is completed. By slowly increasing the temperature, the point at which the strands dissociate can be identified by the subsequent drop in signal as the dye falls from the denatured duplex (8). Because each species present in the reaction will have a characteristic melting profile, it is possible to increase the validity of results obtained using dyes by confirming the presence of a single characteristic melting profile post amplification.

SPECIFIC DETECTION METHODS

Many of the probe-based systems rely on the principle of fluorescence resonance energy transfer (FRET) for signal generation. FRET involves the nonradiative transfer of energy from a donor molecule to an acceptor molecule. The efficiency of energy transfer is proportional to $D \times 10^{-6}$, where D is the distance between the donor and acceptor molecules. When two fluorophores whose excitation and emission spectra overlap are in close physical proximity, excitation of one fluorophore will cause it to emit light at wavelengths that are absorbed by and that stimulate the second fluorophore, causing it to fluoresce. With FRET hybridization probes, a donor fluorophore on one probe is brought into close proximity to an acceptor fluorophore on a second probe when both probes hybridize to adjacent regions on a target molecule (Fig. 1). The donor fluorophore is excited by the light source of the

instrument, and energy is transferred from the donor to the acceptor, producing an increase in measured fluorescence from the acceptor fluorophore.

Other probe-based systems, such as TaqManTM probes (9), Molecular Beacons (10), and ScorpionTM primers (11), rely on the close proximity of donor fluorophore and non-fluorophore acceptor molecules (quenchers) in the unhybridized probe conformation such that little or no signal is generated as the fluorescence of the donor is quenched without subsequent emission of fluorescence by the acceptor. Upon hybridization to the target, the fluorophore and quencher become separated through either conformational changes that occur upon hybridization (Molecular Beacons and Scorpion primers) or enzymatic cleavage of the fluorophore from the quencher upon hybridization as a result of the 5' nuclease activity of *Taq* polymerase (TaqMan probes). The physical separation of the fluorophore and quencher moieties produces an increase in signal.

TaqMan probes consist of a probe sequence labeled at one end with a fluorophore and at the other end with a quencher moiety. In the unhybridized state, fluorescence is quenched because of the close proximity of the fluorophore and quencher. Upon hybridization of the probe to the target sequence during PCR, the 5'-3' exonuclease activity of *Taq* polymerase cleaves the probe; the subsequent separation of the fluorophore from the quencher produces a concomitant increase in fluorescence (Fig. 2).

Molecular Beacons consist of a probe held in a hairpin loop structure by complementary sequences on the 5' and 3' sides of the probe. Fluorophore and quencher moieties on the 5' and 3' ends of the probe are held in close proximity in the unhybridized state. Upon hybridization

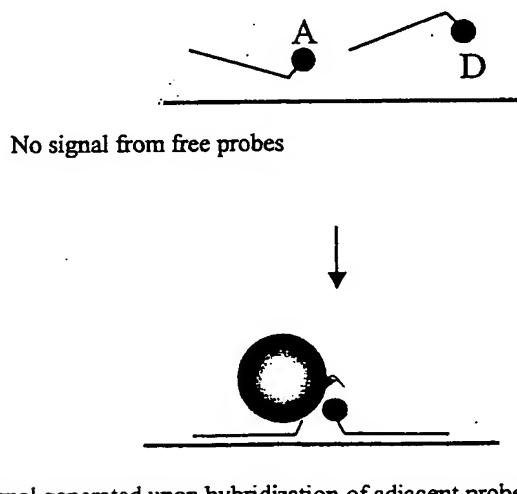


Fig. 1. FRET probes.

Donor (D) and acceptor (A) fluorophores are linked to hybridization probes. Upon hybridization to target sequences, the donor and acceptor fluorophores are brought into close proximity. The energy absorbed by the donor fluorophore is transferred to the acceptor fluorophore and emitted as fluorescence of a different wavelength. In isolation, the acceptor fluorophore is not excited and does not fluoresce.

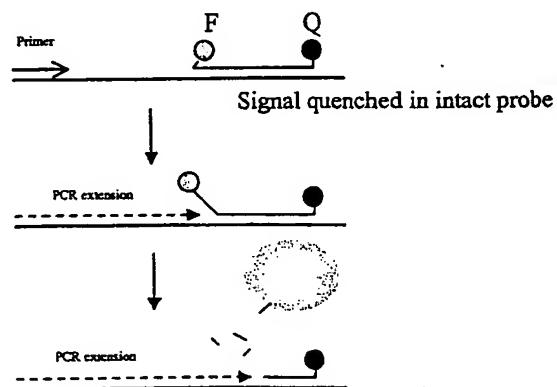


Fig. 2. TaqMan probes.

The fluorophore (F) linked to intact TaqMan probes is efficiently quenched by the quencher (Q) moiety. TaqMan probes hybridize to DNA targets during PCR extension. When *Taq* DNA polymerase reaches the hybridized oligonucleotide, it causes strand displacement of the TaqMan probe. The 5'-3' exonuclease activity of *Taq* promotes cleavage of the probe between fluorophore and quencher components, spatially separating the two moieties. Once separated from the quencher, the fluorophore emits fluorescent signal [adapted from Livak et al. (9)].

to the target sequence, the loop opens out; the resulting separation of the fluorophore from the quencher produces an increase in signal (Fig. 3). Molecular Beacons were reported to have a higher specificity than TaqMan probes because of the presence of a stem structure (10). Hybrids between the beacons and mismatch targets dissociate at lower temperatures than do hybrids between linear probes and mismatch targets. Thus, a wider temperature range is observed between melting of perfect and mismatch hybrids.

Scorpion primers consist of a probe sequence linked to the 5' end of a primer via a nonamplifiable stopper moiety. The probe is held in a hairpin loop structure by complementary sequences on the 5' and 3' sides of the probe. A fluorophore at the 5' end of the probe is quenched by a moiety at the 3' end of the loop in the unhybridized format (similar to Molecular Beacons). As the primer extends during PCR, the probe sequence is capable of hybridizing to a target on the newly formed strand. Upon hybridization, the loop is opened, producing a physical separation of the fluorophore and quencher such that increases in signal are observed (Fig. 4).

Probe-based systems can be used for both quantitative and qualitative applications. For qualitative applications, the probes are designed to hybridize to the region containing the specific sequence or mutation under investigation. Amplification is performed using an annealing temperature intermediate to the melting temperature (T_m) of the probe on its matched and mismatched targets such that only when the probe perfectly matches the target will signal be generated.

For quantitative applications, comparison of the sample C_t to a calibration curve or the C_t of a suitable reference reaction is required. The reference used will depend on whether relative or absolute quantification is required and will be discussed in more detail later.

Clinical Applications of Real-Time PCR

Real-time PCR methods have been described for a variety of clinical applications; these include genotyping, patho-

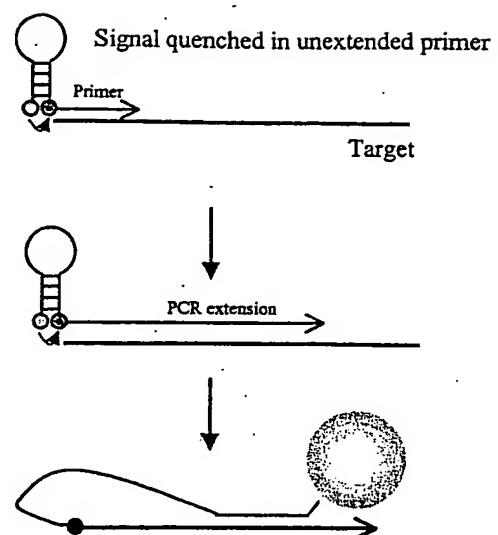


Fig. 4. Scorpion primers.

In isolation, Scorpion primers are fully quenched and do not fluoresce. However, following hybridization to amplified target sequences, the fluorophore (O) becomes spatially separated from the quencher (●) and emits significantly greater amounts of fluorescent signal [adapted from Whitcombe et al. (11)].

gen detection, and viral load assessment, reverse transcription-PCR and mRNA quantification, oncology, and residual disease monitoring and immunology.

GENOTYPING

Bon et al. (12) describe the genotyping of the HLA-B27 allele using allele-specific primers, SYBR Green I, and the LightCycler. Using this technique, the authors were able to correctly genotype 100 individuals previously characterized by nonhomogeneous methods. The authors highlighted the need for stringent optimization of PCR conditions such that the internal control β -globin product was always produced in HLA-B27-negative samples, but at the same time, did not outcompete the HLA-B27 product in positive samples.

A polymorphism in exon 6 of the paraoxonase gene and one in the human apolipoprotein B gene were used as model systems in a different approach, based on allele discrimination by postamplification melting curve analysis assayed on the ABI GeneAmp 5700 Sequence Detection System using SYBR Green I (13). Amplification products were discriminated by the addition of a 5' GC-tail to one of the allele-specific primers such that allele-specific amplicons had significantly distinct T_m s. With this homogeneous assay, 100 samples were successfully genotyped for polymorphisms in the paraoxonase gene and the apolipoprotein B gene.

FRET probes were used to detect the α_1 -antitrypsin deficiency alleles Pi^*Z and Pi^*S (14). Probes designed to hybridize over the variant region were synthesized with the probe being perfectly matched to the wild-type sequence and mismatched at the central base in the presence

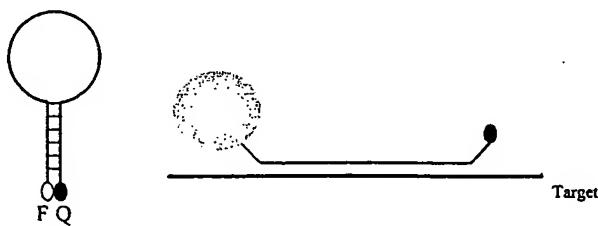


Fig. 3. Molecular Beacons.

In unhybridized Molecular Beacons, the stem-loop structure of the probe brings fluorophore (O) and quencher (●) moieties into close proximity, such that the fluorophore is quenched and the probe does not fluoresce. When molecular beacons hybridize to target DNA sequences, the fluorophore and quencher components become spatially separated and the probe fluoresces [adapted from Tyagi and Kramer (10)].

of the polymorphism. Allele discrimination was demonstrated in real time by use of an annealing temperature during PCR that was intermediate to the T_m of the probe on the matched and mismatched targets. Postamplification melting curve analyses also allowed allele discrimination, with the sensor probe on the mismatched target melting at a lower temperature (with a subsequent reduction in signal) than that for the matched target. More than 50 individuals were typed, and all genotypes were in agreement with those obtained by alternative methods (PCR-restriction fragment length polymorphism analysis).

The same group has also used the same approach to detect apolipoprotein E isoforms, the apolipoprotein B3500 mutation (15), a prothrombin mutation, and the C677T mutation in the methylenetetrahydrofolate reductase (*MTHFR*) gene (16). Similar approaches have also been used to detect mutations in the factor V Leiden gene (17), the cystic fibrosis transconductance regulator (*CFTR*) gene (18), the hemochromatosis gene (19, 20), and the human platelet antigen 1 gene (21).

TaqMan chemistry currently is the most established of all the homogeneous detection systems. The application of TaqMan chemistry for the detection of genetic alterations has been widely reported. Recent examples include the detection of mutations in the prothrombin and *MTHFR* genes (22) and the factor V Leiden mutation (23) where probes specific for each allele labeled with different fluorophores [6-carboxyfluorescein (FAM) and tetrachloro-6-carboxyfluorescein (TET)] are used in the same reaction.

In other approaches, TaqMan probes have been combined with allele-specific primers. Mutant and wild-type alleles are distinguished by differences in the C_t observed when allele-specific PCR is performed. The TaqMan probe is used as an indicator of amplification. Screening for inherited metabolic disorders caused by mutations in the glycogen storage disease type Ia and medium-chain acyl-Co A dehydrogenase deficiency, using this approach, has been performed (24).

In similar approaches, allele-specific primers and TaqMan probes have been used to detect polymorphisms in drug-metabolizing enzymes (25) and for detection of HLA-DRB alleles (26).

Molecular beacons have been used to detect the C677T mutation of the *MTHFR* gene (27) using the ABI 7700. Wild-type- and mutant-specific beacons were designed and templates assayed with each probe in separate reactions. Annealing temperatures intermediate to those of a beacon on a matched and a mismatched target were used for the real-time PCR assay. The temperature was determined by melting curve analyses of the beacons on matched and mismatched oligonucleotide targets.

Scorpion primers have been used to detect five common cystic fibrosis mutations (28). Probe sequences designed to cover the five polymorphisms were synthesized, and the annealing temperatures initially used during PCR on the LightCycler were those predicted from the mfold modeling

program (<http://bioinfo.math.rpi.edu/~zukerm/>) to be the temperatures at which the probe would be bound to the matched amplicon but dissociated from the mismatched amplicon. Postamplification melting curves were also generated for greater accuracy, and the T_m s obtained from the melting curves were used in subsequent amplifications. All Scorpion primers were able to discriminate between wild-type and mutant samples, although differing discrimination efficiencies and signal strengths were seen between the five assays. All samples were also analyzed with the SYBR gold dsDNA-binding dye in separate capillaries to control for amplification failures. Separate capillaries were required because the FAM dye on the Scorpion primer and SYBR gold are monitored on the same channel. The authors of the study acknowledge that this is a less valid control than one performed in the same capillary as the test assay (28). They also acknowledge the potential constraints of the system, such as the requirement for the close distance between the probe target and Scorpion primer binding site. However, the advantages of the system, such as the speed of signaling of the unimolecular reaction and the reported increased specificity caused by the close proximity of the probe to the target, may outweigh the design constraints.

PATHOGEN DETECTION

FRET probes and the LightCycler have been used for the detection of varicella-zoster (29) and herpes simplex viral particles (30). With the varicella-zoster assay, sensitivity was found to be increased compared with cell culture assays.

A competitive PCR assay has been compared with TaqMan probe assays developed on the ABI 7700 for obtaining quantitative estimates of *Mycobacterium tuberculosis* DNA in sputum (31). Both techniques were found to be reproducible and accurate.

TaqMan probes have also been used for the detection of hepatitis B viral (HBV) particles in patients with chronic HBV infections (32). With the ABI 7700, the TaqMan assay was found to have a dynamic range of $373\text{--}10^{10}$ genome copies/mL. The assay was validated using the Viral Quality Control panel (CLB, Amsterdam, The Netherlands), and the intra- and interassay CVs (of C_t) were reported to be 1.65% and 1.85%, respectively. The authors discussed validation issues surrounding the assay and concluded that the testing of two independent isolates would allow the easier identification of false-positive results. In addition, the inclusion of a suitable internal control in the assay was cited as a critical factor in demonstrating the presence of inhibitory factors in the sample.

TaqMan probes have also been used to type and subtype influenza viruses in respiratory samples (33). The specificity of the assay was examined on 86 influenza A and 49 influenza B reference strains and isolates. The

TaqMan assay was found to be more sensitive than culture methods.

In further studies, TaqMan probes have been used for the detection of meningococcal DNA using the ABI 7700 (34). The assay was compared with equivalent PCR-ELISA assays, and cerebrospinal fluid, plasma, and serum samples were examined. TaqMan probes specific for the meningococcal capsular transfer gene (*ctrA*), insertion seq IS1106, and the sialyltransferase gene (*siaD*) were designed for serogroup B and C determination. The TaqMan assay for IS1106 gave false-positive signals from several nonmeningococcal isolates, whereas the other two assays were specific. The TaqMan assays were found to be more sensitive than ELISA-based assays when culture-confirmed cases were analyzed. After further optimizations, the sensitivities of the TaqMan assays were 93% and 91% for the *ctrA* and *siaD* assays, respectively, compared with culture.

TaqMan assays have also been used to monitor cytomegalovirus (CMV) infections after stem cell transplantation (35) and for the detection of HBV in serum samples (36). In the study of CMV infections, TaqMan assays were compared with a commercially available method [COBAS Amplicor CMV monitor (CMM)]; good correlation was seen between the two detection formats, although the sensitivity of the TaqMan assay appeared slightly compromised compared with the CMM method. Throughput with the TaqMan assay was approximately three times higher than with the CMM method. In the study of HBV, the TaqMan assay was found to be highly reproducible and sensitive, with virus accurately detected in samples containing 10–10⁹ viral copies/mL.

ONCOLOGY

TaqMan probes and the ABI 7700 have been used to detect the t(14;18)(q32;21) translocation in 135 non-Hodgkin lymphomas, 6 Hodgkin disease samples, 10 reactive biopsy specimens, and 11 peripheral blood samples (37). The TaqMan assay was able to detect the translocation in samples serially diluted up to 10⁻⁵-fold. Real-time and conventional PCR assays were compared, and overall concordance was found to be 98.8% (160 of 162 samples). In both discrepancies, real-time PCR picked up the translocation where conventional PCR did not. One case was predicted to be a true positive, whereas the other may have represented a case of reactive follicular hyperplasia with the t(14;18).

The LightCycler has been used for the quantification of minimal residual disease in childhood acute lymphoblastic leukemia (38). The presence of minimal residual disease was detected using T-cell receptor and immunoglobulin gene rearrangements as clonal markers. Quantitative PCR performed with TaqMan probes, hybridization probes, SYBR Green I binding, and standard PCR with oligonucleotide probing were all compared.

bcr/abl fusion transcripts in chronic myelogenous leukemia (CML) were detected using TaqMan probes and the

ABI 7700 (39). In this assay the β -actin gene was used as the quantification control gene. All TaqMan assays quantified down to ~10 copies/100 ng of total cDNA. Clinical samples from nine documented CML cases were examined. Relapsing CML correlated with increasing concentrations of *bcr/abl* fusion RNA transcripts, whereas decreasing concentrations were seen in those patients successfully treated with antileukemic treatment. In another study, the authors examined the same assay on the LightCycler instrument (40) and found that the assay produced comparable results in terms of sensitivity and efficiency on both platforms.

In a similar approach, Barbany et al. (41) also looked at the detection of *bcr/abl* transcripts in CML patients using TaqMan probes. In this study, *GAPDH* was used as the quantification control gene. The detection limit was reported to be 1 positive cell among 10⁵ negative cells. The real-time data were found to correlate well with cytogenetic data, with the interassay variation of the method (CV) being 32%.

In a slightly different approach, allele-specific primers and allele-specific TaqMan probes were used for the analysis of the complementary determining region 3 (CDR3) of the rearranged immunoglobulin heavy chain gene (*IGH*) in patients with multiple myeloma (42). Minimal residual disease was detected and quantified in bone marrow samples from multiple myeloma patients undergoing peripheral blood stem cell transplantation. The method was able to detect malignant cells in patients considered to be in remission.

Validation Issues

SPECIFICITY

Assay specificity can be defined as the ability to detect a particular analyte in a complex mixture without interference from other components in the mixture. With real-time PCR assays, specificity can be seriously compromised if suboptimal assay conditions are used.

The ability to discriminate single base differences is an important feature of many assays. The length of the probe, position of mismatch within the probe, fluorescence acquisition temperature, and magnesium chloride concentration of the buffer are all factors that can affect the ability of the assay to detect single base changes.

The design of suitable probes for performing real-time analyses is still not a trivial task, as many of the techniques require secondary structures to be designed into the probes. Software packages to predict nucleic acid secondary structures are available and include Primer Express (ABI/PE Biosystems), Gene Works (IntelliGenetics), and Oligo (National Biosciences, Inc). Guidelines for successful probe design are also available from the system manufacturers, such as ABI and Roche. Probe and assay design criteria for quantitative PCR applications using the 5'-nuclease assay have recently been reviewed (43). Even with the help of prediction software, assay optimization can be time-consuming and expensive, and the use of

H₂NH₂NH₂H₂O

$$3 \text{ pg} = 1 \text{ c.u.}$$

$$3 \text{ ng} = \underline{1000 \text{ c.u.}}$$

additional techniques during assay development such as agarose gel electrophoresis often are required to ensure that specific amplicon is produced.

Molecular Beacons are reported to be more specific than TaqMan probes because of the stem-loop structure of the beacons, which destabilizes the hybridization of the probe to a mismatched target. Scorpion primers are reported to be more specific still because the stem-loop probe is held in close proximity to the target by virtue of being attached to the PCR primers.

One study examined the performance of two different technologies (TaqMan probes and Molecular Beacons) to detect three single nucleotide polymorphisms (SNPs) in the human estrogen receptor gene (44). The study concluded that for two of the three SNPs, similar discriminatory power was achieved with both technologies. However, the Molecular Beacons produced more reliable genotyping results in the GC-rich target region than the TaqMan probes.

Experimental evidence for the differing specificity has been provided by relatively few studies; further studies will be required to determine whether the differences are universal features. The availability of reference panels of well-characterized samples will aid assay design. In the clinical laboratory, the use of "home brew" assay formats often makes comparisons between laboratories difficult. Proficiency testing schemes and ring trials go some way to ensuring that individual assays are fit for the purpose for which they are intended for specific applications and that the staff performing them are competent.

The authors of a genotyping study using a real-time homogeneous melting curve assay (13) considered specificity as a validation issue and highlighted the importance of assay optimization to minimize nonspecific amplification. Hot-start PCR, the polymerase enzyme chosen, salt concentration, and the annealing temperature during PCR were all cited as critical factors. The use of Stoffel enzyme was reported to enhance discrimination of 3' primer-template mismatches. The possibility of false genotypes being generated as a consequence of very low initial copy number of template was also discussed, and the authors suggested setting a cycle limit and discounting all reactions with C_ts occurring later than the limit. The authors further state that it may be difficult to optimize the assay for very GC-rich targets because the assay relies on the discriminatory effect of the GC-tailed primer. The need for careful optimization of the Mg²⁺ concentration was also highlighted by other authors (12) as a critical point for the discrimination of products by melting curve analysis.

SENSITIVITY

The sensitivity required for an assay is dependent on the rarity of the target molecule in the sample. In many situations, detection limits approaching the single molecule level are desirable. For example, in the field of oncology, a mutation may be present in a single cell in a

background of thousands of "normal" cells. In virology, only one cell out of thousands may be infected.

For assays requiring very high sensitivity, sensitivity can be compromised by several factors, including the presence of competitors and inhibitors in the sample and interference of the background fluorescence of the system. The major source of background fluorescence arises from the incomplete quenching of the probes in their native state. Background fluorescence also arises from a combination of the physical detection process, the nonspecific signals from undesired sample components, and the signals from specific contaminants or competitors. According to the manufacturer (45), with the Roche LightCycler system and hybridization probes, specific detection of the single-copy β -globin gene was achieved from 3 pg of human genomic DNA (~1 human genome equivalent). The same report stated that for other targets, a slightly lower sensitivity was achieved (3–30 pg).

Beacons and Scorpions are reported to be more efficiently quenched than TaqMan probes in the unhybridized state because the stem-loop structures designed into the probes hold the fluorophore and quencher together in very close proximity. TaqMan probes do not benefit from such secondary structures, and therefore generally are reported to have a higher background fluorescence, which can compromise assay sensitivity.

The ability to detect one allele present as a minority in a mixed sample has been examined by comparing TaqMan probes and Molecular Beacons using three SNPs in the human estrogen receptor gene (44). Detection limits varied between the SNPs, but were found to be similar for both assays, with the range being 1.6–25% (percentage of minority allele in total sample).

For many assays, arbitrary cutoff points for calling samples as positive and negative are used. This can produce wide variability among laboratories. The highly sensitive nature of many of the assays can have implications for accurate diagnosis. In a study to detect varicella-zoster by real-time homogeneous analysis, Espy et al. (29) designed assays to detect two genes and found that one assay had a higher sensitivity than the other. The authors recognized the possibility of false positives occurring at such high sensitivities and recommended that only samples positive for both genes be counted as genuinely positive.

In the field of pathogen detection, it may not be possible to readily distinguish carriers from those with clinically apparent infections. TaqMan probes were used to detect *Mycoplasma pneumoniae* infections in clinical samples (46). Thirty-one patients were positive by PCR, but only 23 were positive by serology. No patients were positive by serology and negative by PCR. The discrepant results may have indicated false-negative serology findings or may have represented carriers of *M. pneumoniae* rather than clinically affected individuals. This study highlighted the difficulty of using extremely sensitive assays in distinguishing carriers from patients. The setting

of appropriate cutoff Ct values to reliably distinguish the two groups will require a considerable amount of effort.

Consensus of meaningful cutoff values will need to be established between laboratories. The use of standardized protocols and chemistries will help in interlaboratory comparisons. Widely available standard reference panels of accurately quantified samples will help to establish the sensitivities of assays among laboratories.

REPRODUCIBILITY

At very low DNA target concentrations, the variability will be greater because of stochastic variations in target amplification. A recent report analyzing the intraassay CVs of the Roche LightCycler for the quantification of varying starting copies of template found that for 15 replicates, the CV (of copy number) was 5.9% for 10^5 copies, 8.8% for 10^3 copies, and 41% for 10 copies (47). This is exemplified by a study on translocations in Hodgkin lymphoma (37). The TaqMan-based assay was able to detect the translocation in samples serially diluted up to 10^{-5} -fold. However, as may be expected, the sequence was inconsistently detected in 10^{-5} -fold dilutions because this represents 0.6–0.8 genome copies. The authors reported that accurate quantification could be obtained by generating calibration curves from serial dilutions of positive cell lines.

For clinical samples, replicates will be essential, especially if a low target copy number is expected.

Many studies have reported the intra- and interassay variability for homogeneous assays, but studies detailing interlaboratory variability in this area are lacking. As discussed above, the availability of standardized protocols, reference materials, and chemistries as well as standardized template processing protocols will help to limit interlaboratory variability.

ROBUSTNESS

There is evidence to suggest that the polymerase enzymes used in homogeneous assays will have an impact on performance. This is of particular importance for TaqMan assays, which rely on the 5'-exonuclease activity of *Taq* polymerase for signal generation. A recent study demonstrated that the polymerase and 5'-exonuclease activities of a range of commercially available enzymes varied significantly (48). Fifteen commercially available enzymes were investigated using a TaqMan probe format. The Cts and shapes of the amplification curves were demonstrated to vary substantially, and three enzymes with reported 5'-exonuclease activity failed to produce any fluorescence signal.

The performance and reproducibility of the various technologies across different platforms have not been widely examined. The differing speeds of thermal cycling between platforms are likely to have an impact on assay performance because the reaction kinetics of the various technologies vary. The hybridization kinetics of techniques that rely on unimolecular probe/target interac-

tions, such as Scorpion primers, are very rapid and may therefore benefit from faster cycling, which may help to reduce background and the production of nonspecific species (28). Techniques that rely on bimolecular reactions, such as Molecular Beacons, are kinetically slower and may not perform as well under ultrarapid cycling conditions, whereas techniques that rely on enzymatic degradation for signal generation, such as TaqMan, may also benefit from slightly slower cycling.

A report by Kreuzer et al. (40) indicated that the TaqMan chemistry used for the detection of *bcr/abl* transcripts performed equivalently on both the ABI 7700 and LightCycler platforms. Another study by the same group (49) demonstrated that TaqMan probes designed to quantify CMV viral load also performed equivalently on both the ABI 7700 and LightCycler platforms. In addition, with the LightCycler platform, TaqMan probes were replaced with FRET hybridization probes without a loss in sensitivity or reproducibility. A wider range of assays will need to be examined on different platforms to determine the overall robustness of the various chemistries.

One study compared Scorpion, TaqMan, and Molecular Beacon chemistries using the LightCycler and indicated that each chemistry does perform slightly differently (28). The unimolecular action of the Scorpion primers was shown to occur faster and more efficiently than the bimolecular reactions (Beacons and TaqMan).

FRET hybridization probes, Beacons, and Scorpions allow the generation of melting curve data because the signal generated is dependent on hybridization of the probe to the target, which is a reversible process. The generation of melting-curve data can act as a confirmatory tool for many real-time PCR assays.

Commercial methods based on FRET hybridization probes for use with real-time PCR instruments are starting to become available (Roche Diagnostics). One such method designed for the detection of mutations in the apolipoprotein B gene has recently been evaluated (50). The method was found to be robust and accurate. Predeveloped TaqMan reagents are also available for the detection of several cytochrome P450 polymorphisms (<http://www.appliedbiosystems.com>).

Data Interpretation

Another issue in assay performance is data interpretation where there often is a lack of quality measures. The software must be able to distinguish between genuine signals and erroneous artifacts. Accurate identification of baselines and Cts is critical for the generation of meaningful data. The ability to store, track, and retrieve data accurately and make comparisons between experiments is currently being addressed by several companies. The ability to view the output data manually to highlight any anomalies is a benefit, although with such large amounts of data generated, identification of problems by eye may not always be possible.

The multiplexing of probes labeled with different flu-

orophores can cause problems unless the data are normalized to take into account differing dye fluorescence intensities and spectral cross-talk. A study using multiplexed FRET hybridization probes for the detection of apolipoprotein E polymorphisms (51) developed algorithms to compensate for the temperature-dependent spectral overlap of multiple fluorophores.

Controls

No data generated by PCR can be assumed to be valid in the absence of suitable positive and negative controls. The risk of contamination is reduced by the use of closed-tube homogeneous assays, but it can never be totally eliminated. As with all PCR assays, negative PCR controls are required to detect possible contamination.

During a run, well-to-well variation can be normalized by use of a reference fluorescent dye. This could be the quencher fluorophore, which remains constant throughout amplification.

For assays where quantification is required, the ratio of specific signal to that obtained from a constant housekeeping control gene often is used. Careful selection of the housekeeping gene is required to ensure that the gene chosen is truly invariable under all experimental conditions.

For absolute quantification measurements, calibration curves are often generated from serial dilutions of known amounts of analyte. Reviews of the application of quantification calibrators typically used in real-time analyses have recently been published (52, 53).

For genotyping assays, control samples of known genotype are required, and centrally available stocks of validated samples will assist in new assay development and the continued quality assurance of clinical assays.

Conclusion

Recent advances in DNA-based technologies and instrumentation have led to the development of assays whereby DNA targets can be amplified and analyzed in a single closed tube in minutes rather than hours. The benefits of these assays in terms of increased throughput, reduced risk of contamination, increased sensitivity, and easier automation of assays, which require little manual handling, are obvious. However, these newer technologies pose many challenges to the clinical diagnostic setting where the interpretation of the data generated can be affected by many factors (54). Critical factors include the inherent differences of the newer chemistries in terms of specificity, sensitivity, and robustness, which can in turn be compounded by the instrumentation chosen. The cut-offs to distinguish positive from negative samples, the assay controls and quantification calibrators used, and the ability to accurately distinguish specific from nonspecific products can all influence interpretation of results.

Before these technologies can become widely used in the clinical setting, there is a need for greater standard-

ization of the methods used and interlaboratory validation of the technologies.

In general, test regulation occurs through accepted practice rather than performance standards. Interlaboratory variability in the ability to correctly genotype a panel of test samples using more traditional DNA-based techniques was highlighted in a recent study evaluating the quality of cystic fibrosis testing in laboratories across Europe (55). Less than one-half of the laboratories made no errors in the genotyping of six reference samples during three consecutive annual trials. The participants used one of a range of strategies for genotyping, including commercial and home-brew methods. The cause of incorrect results included technical reasons, misinterpretation of results, and reporting errors.

Sharing of the technical information for assay design and sample preparation will help. The provision of validated and accurately quantified calibrators and controls will allow interlaboratory comparisons to be made.

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